Neuroendocrine Gene Regulation and Body Weight Control: FoxO1 Protein and Transcriptional Regulation

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Abstract: The insulin-regulated transcription factor FoxO1 is a central player in metabolism that integrates various environmental and metabolic cues to generate dynamic gene expression programs. This review focuses on the molecular mechanisms involved in FoxO1's effects in hypothalamic neurons and the inherent implications for physiopathologic processes in energy homeostasis. Particular emphasis is placed on the emerging body of evidence pointing to a multi-layered control of melanocortinergic tone by FoxO1 in concert with the leptin-induced transcriptional activator Stat3.

EXPRESSION AND REGULATION OF FOXO1 ACTIVITY IN THE HYPOTHALAMUS

Hypothalamic neurons are direct targets of several metabolic stimuli whose action is required to maintain energy balance. Key signals communicating information about the body's peripheral energy stores to the hypothalamic control center include the pancreatic hormone insulin and the adipocyte-derived hormone leptin. Initiation of leptin receptor signaling activates the signal transducer and activator of transcription (Stat) 3 [1]. Cytokines, growth factors, and hormones like insulin control transcriptional activity of the three isoforms of forkhead box-containing proteins of the O subfamily (FoxOs). This is achieved by shuttling FoxOs to either the nucleus or the cytoplasm. Insulin receptor (InsR) signaling results in phosphorylation, nuclear exclusion and proteosomal degradation of the transcription factor FoxO1 via activation of the Pi3-kinase (Pi3k)/Akt pathway [2-4].

In the mouse, FoxO1, FoxO3, and FoxO6 are expressed in the central nervous system (CNS), but only FoxO1 and FoxO3 appear to be regulated by phosphorylation [5]. Expression of the main isoform FoxO1 is first detected in mid-gestation in the developing mouse brain around E14.5. In the adult CNS, FoxO1 is abundant in the hippocampal dentate gyrus, the amygdalo-hippocampal region, the piriform cortex, the striatum, and the hypothalamus [5,6]. FoxO1 protein is present in the majority of cells in key hypothalamic nuclei involved in energy homeostasis, i.e., the dorsomedial (DMH), ventromedial (VMH), and arcuate (ARC) hypothalamic nucleus. In the latter, FoxO1 is particularly abundant in two neuronal subpopulations characterized by expression of pro-opiomelanocortin (POMC) or agouti-related protein (AgRP) [6].

Regulation of FoxO1 activity is complex. In addition to Pi3k, several other centrally expressed kinases including serum- and glucocorticoid-inducible kinase 3 [7], dual-specificity tyrosine-phosphorylated and regulated kinase [8], and cyclin-dependent kinase 2 [9] phosphorylate FoxO1, pointing to its role as a master integrator that coordinates inputs from various signaling pathways. While a considerable amount of evidence highlights the pivotal role of hypothalamic Pi3k signaling in the regulation of glucose and energy homeostasis by both insulin and leptin [10,11], the role of the other kinases in this regard has not been elucidated.

In addition to these kinases, it is interesting to note that activation of adiponectin receptor 1 in the ARC by the insulin-sensitizing cytokine adiponectin utilizes the canonical InsR/Pi3k/FoxO1 signaling pathway to decrease feeding [12]. Pi3k-mediated homeostatic effects of insulin and leptin at the molecular level further include regulation of membrane potential by K_{ATP} channels and actin reorganization [11], but hypothalamic 3-phosphoinositide-dependent protein kinase 1 (Pdk1), a downstream target of Pi3k, appears to control energy homeostasis mainly through regulation of FoxO1 activity [13].

Other mechanisms by which FoxO activity is modulated post-translationally include ubiquitinylation and acetylation. Environmental stimuli like oxidative stress events lead to FoxO1 acetylation through the histone acetyl-transferases CBP and p300; this process is reversed by the actions of several histone deacetylases, the most studied of which is the NAD-dependent deacetylase Sir2 [14-16]. Even though the functional role of FoxO1 acetylation and deacetylation on the initiation of specific transcriptional programs is still a matter of debate, the bulk of evidence indicates that FoxO1 acetylation—while diminishing transcriptional activity—prevents ubiquitin-dependent degradation [17]. Furthermore, the relevance of this mechanism to the regulation of FoxO1 activity in hypothalamic neurons is unknown. A previous study analyzing the metabolic effects of a ubiquitously expressed Sir2α gain-of-function variant in mice has been suggestive of a role of hypothalamic Sir2 in energy homeostasis through regulation of FoxO1 acetylation [18], but knowledge about the underlying mechanisms remains limited.

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Interestingly, a very recent study has demonstrated that the anorexigenic effects of insulin, when applied by direct intracerebroventricular administration, are dependent on hypothalamic reactive oxygen species (ROS) production through NADPH oxidase [19]. Further, it has been reported that FoxO1 inhibition correlates with an increase in reactive oxygen species (ROS) in adipocytes, and was reversed upon treatment with the weak Sir2 agonist resveratrol, which increased FoxO1 protein levels in adipocytes and concomitantly decreased generation of ROS [20]. In adipocytes, an early burst of ROS production by insulin is required for its downstream signaling, including activation of the Pi3k pathway, and is in part mediated by oxidative inhibition of protein tyrosine phosphatases (like PTP1B and PP2A) that normally antagonize insulin action [21]. Within the hypothalamus, not only insulin-induced ROS production but also Pi3k signaling are essential for insulin's anorexigenic effect [19,22]. Given these analogies between intracellular signaling events in adipocytes and hypothalamic neurons, it is tempting to speculate that FoxO1, together with Sir2, is involved in the regulation of insulin-mediated hypothalamic ROS production. Further studies are warranted to establish a defined molecular link between metabolic and stress pathways in hypothalamic neurons.

METABOLIC SENSING BY FOXO1 IN HEALTH AND DISEASE CONDITIONS

Consistent with FoxO1's metabolic sensing role in the hypothalamus, studies on organotypic slices with FoxO1-GFP-expressing POMC or AgRP neurons demonstrated Pi3k-dependent nuclear exclusion of FoxO1-GFP protein upon treatment with either insulin or leptin [23]. Accordingly, FoxO1 subcellular localization in ARC neurons changes dependent on nutritional status, with predominantly nuclear localization during fasting and mostly cytoplasmic localization upon feeding [6,23]. In line with the finding of transcriptional inactivation of FoxO1 by the anorexigenic hormones insulin and leptin, ARC-specific inhibition of FoxO1 causes hypersensitivity to the anorexigenic effects of leptin and hypophagia, while over-activation induces leptin resistance and hyperphagia [6,24-26]. In this regard, it is interesting to note that diet-induced obesity (DIO) is associated with impaired hypothalamic InsR signaling owing to reduced activation and increased proteosomal degradation of FoxO1 [26], a phenomenon that has similarly been observed in organotypic hypothalamic slices *in vitro*, in which pretreatment with free fatty acids blocks insulin-induced FoxO1-GFP nucleo-cytoplasmic translocation in POMC neurons [23]. As a consequence, centrally applied insulin possesses reduced anorexigenic potency in DIO rodents. Conversely, mice with POMC-specific genetic ablation of FoxO1 ($Foxo1^{\Delta POMC}$) exhibit resistance towards adult-onset DIO [25]. Taken together, these findings suggest that inactivation of hypothalamic FoxO1 is required for the full anorexigenic response elicited by insulin and that fatty acid-induced insulin resistance is associated with impaired FoxO1 nuclear export in hypothalamic neurons, implicating FoxO1 and its targets in these cells in the pathogenesis of metabolic diseases.

Nonetheless, there remains some controversy about whether the homeostatic effects of InsR/FoxO1 signaling play a role under basal physiological conditions or only during metabolic challenge or disease states. It should be noted that virtually all studies that have demonstrated an acute effect of insulin on food intake were performed in fasted animals [19,22,27-29]. In this situation, the hypothalamic system is challenged by food deprivation with insulin at nadir levels and mostly nuclear localization of FoxO1. *In vivo* studies investigating the homeostatic effects of FoxO1 have led to inconclusive results, most likely due to different experimental models and conditions. While Kim *et al.* [24] reported decreased food intake and body weight in lean mice upon siRNA-mediated knock-down of ARC *Foxo1*, Ropelle *et al.* [26] could not detect an effect of ASO-induced FoxO1 ablation on cumulative food intake of lean animals, but showed a marked anorexigenic effect of FoxO1-ASO in DIO rats. In our own studies, injection of a dominant-negative FoxO1 mutant prevented the fasting-induced increase in expression of the orexigenic neuropeptide AgRP, but did not affect AgRP expression under basal conditions. Along these lines, basal food intake in *Foxo1* haploinsufficient mice was comparable to wild type controls and differed only after intracerebroventricular delivery of leptin [6]. On the other hand, $Foxo1^{\Delta POMC}$ mice exhibit significant hypophagia not only after leptin treatment and during fasting-provoked refeeding, but also under basal conditions [25]. Finally, adenovirus-mediated overexpression of constitutively nuclear FoxO1 readily induces hyperphagia and weight gain under *ad-libitum* feeding conditions [6]. Taken together, these results suggest that adequate regulation of FoxO1 is indispensable for feeding control, but fail to address the question as to the physiological cues regulating FoxO1 activity. Complicating matters, the direct orexigenic effects of hypothalamic FoxO1 lead to increased body weight and fat mass. This may in turn indirectly cause resistance to insulin and leptin in hypothalamic neurons, thus impeding the assignment of specific phenotypes to direct FoxO1 actions. Further investigation is needed to dissect direct from indirect FoxO1 effects in health and disease conditions.

MULTI-LAYERED CONTROL OF MELANOCORTINERGIC TONE

We and others have demonstrated that FoxO1 functions as a major regulator of melanocortinergic tone at the molecular level [6,13,24,25] (Fig. **1**). Existing data indicate that this is achieved by multi-layered control at the developmental, transcriptional, and post-transcriptional levels. While a role of InsR/FoxO1 signaling in the development of hypothalamic feeding circuits and ARC neurons is evident [25,30] (Plum/Lin/Accili, unpublished data), knowledge about the underlying mechanisms remains vague. Many studies have investigated the role of the maternal environment in this context, examining maternal nutritional status, fetal insulin and leptin levels [31-33], but the role of InsR/FoxO1 signaling in developing neurons remains unknown.

FOXO1 CONTROLS NEUROPEPTIDE EXPRESSION

In contrast, the transcriptional effects of FoxO1 particularly with respect to hypothalamic AgRP and POMC neuropeptides have been a matter of intense research. It has been shown that FoxO1 is recruited to the *Agrp* (possibly also *Npy*) and *Pomc* promoters, where it acts to induce *Agrp*

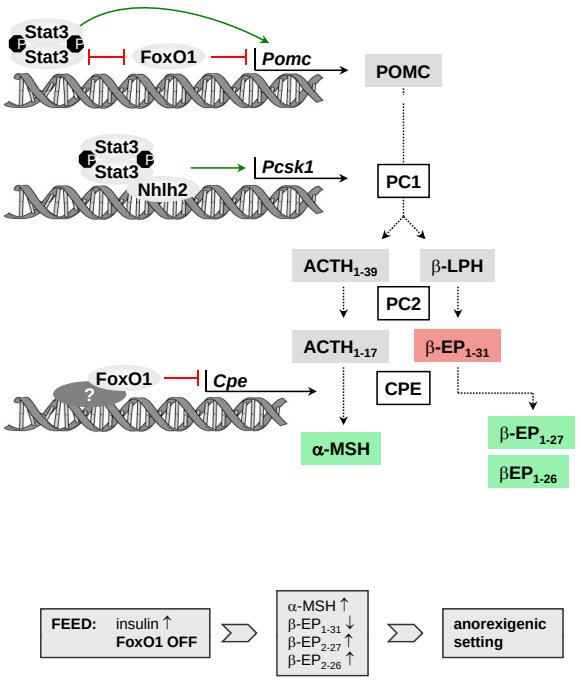

Fig. (1). Regulation of posttranslational processing of hypothalamic POMC by FoxO1 and Stat3.

FoxO1 suppresses (red bar) while activated (phospharylated and homodimerized) Stat3 induces (green arrow) *Pomc* transcription. FoxO1 and Stat3 compete for binding (red bar) at partly overlapping DNA binding sites in the *Pomc* promoter. Stat3 further induces prohormone convertase (*Pcsk*, PC) 1 activity through interaction with the nescient helix loop helix (Nhlh) 2 transcription factor. On the other hand, FoxO1 is recruited to the Carboxypeptidase E (*Cpe*, CPE) promoter. It suppresses *Cpe* expression most likely independent of its ability to bind to DNA; possible interaction partners mediating FoxO1's activity at the *Cpe* promoter are unknown (?). As a result, FoxO1 suppresses while Stat3 stimulates generation of an anorexigenic melanocortin neuropeptide profile. Consequently, inactivation of FoxO1 (e.g., by insulin) disinhibits generation of anorexigenic peptides from POMC.

Prohormone convertase 2: PC2; *Pcsk2*; adrenocorticotrophic hormone: ACTH; beta-lipotropin: β-LPH; alpha-melanocyte stimulating hormone: α-MSH; beta-endorphin: β-EP. Orexigenic peptides are shaded in pink, and anorexigenic (or in the case of β-EP, less orexigenic) peptides in green.

and suppress *Pomc* neuropeptide transcription [6,24] (Fig. **1**). These opposing effects of FoxO1 on *Agrp* and *Pomc* transcription result from its ability to promote coactivator/corepressor exchange at open chromatin. In this context, stimulation of *Agrp* expression is associated with recruitment of CBP/p300 and inhibition of nuclear corepressor (NcoR) and histone deacetylase (Hdac1) binding, whereas the opposite is true for the *Pomc* promoter [6]. Furthermore, FoxO1 and Stat3 appear to compete for binding at the two promoters, providing an explanation for the synergistic anorexigenic effects of insulin and leptin at the transcriptional level. Another study further identified the *Npy* promoter as a possible FoxO1 target and reported inhibitory effects of insulin and leptin on FoxO1-induced *Npy* transcription *in vitro* [24]. But this finding awaits further verification, in light of the fact that mice with disrupted leptin-dependent Stat3 signaling exhibit unaltered *Npy* expression, and mice with ARC-specific FoxO1 gain- or loss-of-function show variable outcomes with respect to *Npy* expression [6,24,34].

EFFECTS OF FOXO1 ON NEUROPEPTIDE PROCESSING

We have recently shown that, in addition to regulating neuropeptide mRNA expression, FoxO1 modulates neuropeptide action by regulation of posttranslational POMC processing [25]. Full-length POMC neuropeptide has to undergo limited proteolysis and other posttranslational modifications to generate several biologically active peptides including α-melanocyte-stimulating hormone (MSH) and β-endorphin (EP). While α-MSH is the main anorexigenic effector peptide at the melanocortin 4 receptor (MC4R) in target regions of ARC POMC neurons [35], $\beta\text{-EP}_{1\text{-}31}$ exerts orexigenic effects via opiate receptor activation. Importantly, further cleavage of $\beta\text{-EP}_{1\text{-}31}$ into $\beta\text{-EP}_{1\text{-}26}$ and $\beta\text{-EP}_{1\text{-}27}$ reduces opioidergic and orexigenic activity of β-EP [36,37]. On the other hand, posttranslational proteolysis of the α-MSH antagonist AgRP is not required for its biological activity [38]. The cleavage process is mediated by several peptidases such as prohormone convertases (PC) 1 and 2 (encoded by *Pcsk1* and *Pcsk2*) and Carboxypeptidase E (Cpe) [39]. Consistent with generation of anorexigenic peptides by POMC cleavage, fasting suppresses expression of hypothalamic *Pcsk1* and *Pcsk2*. Previous studies have shown that leptin-induced Stat3 activation reverses this inhibition by a process involving interaction with the nescient helix-loop-helix (Nhlh) 2 transcription factor [40,41]. Further studies will help to identify a putative role of InsR/FoxO1 signaling for the regulation of *Pcsk1* activity upon feeding and fasting. On the other hand, we could not detect an effect of leptin/Stat3 signaling on *Cpe* activity, but found that FoxO1 suppresses *Cpe* expression in hypothalamic POMC neurons *in vivo* and *in vitro* [25] (Fig. **1**). While ChIP assays show recruitment of FoxO1 protein to the *Cpe* promoter, promoter studies with a DNA binding-deficient FoxO1 mutant suggest that FoxO1-mediated suppression of *Cpe* occurs independent of FoxO1 binding to DNA, i.e. through interaction with other yet unidentified proteins. Interestingly, loss-of-function of *Cpe* causes obesity and diabetes in the *fat* mutant mouse, leading to defects in POMC cleavage with marked reduction of its anorexigenic products in the hypothalamus [42,43]. Conversely, $\text{Foxo1}^{\Delta\text{POMC}}$ mice show increased *Cpe* expression in the ARC, resulting in generation of α-MSH from POMC, and increased cleavage of $\beta\text{-EP}_{1\text{-}31}$ into its less orexigenic forms $\beta\text{-EP}_{1\text{-}26}$ and $\beta\text{-EP}_{1\text{-}27}$, providing an explanation for hypophagia and leanness in these mice [25].

In conclusion, FoxO1 has evolved as a key regulator of energy homeostatic processes not only in peripheral tissues [44] but also in hypothalamic neurons. Its regulation is essential for coordinated control of feeding by insulin and involves a series of tight control mechanisms. The exact nature of these processes, the effectors required and their interplay present a complex puzzle, whose pieces are just beginning to fall into place. Hypothalamic responsiveness to

insulin (and leptin) in the hypothalamus appears to be a key aspect in the development and prevention of obesity [45,46]. Therefore, distinct downstream targets of InsR signaling like FoxO1 may prove attractive candidates for drug development in the treatment of obesity and insulin resistance.

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